"Studies in Natural Products of Biological Interest"

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Date 16.7.1988

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CERTIFICATE

This is to certify that the work embodied in this dissertation has been carried out by Mr.Riaz Ahmad Khan, M.Sc., under my supervision. He has fulfilled the requirements for the degree of Master of Philosophy of the Aligarh Muslim University, Aligarh, regarding the nature and prescribed period of investigational work.

The work included in the thesis is all original, unless stated otherwise, and has not been submitted for any other degree.

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R. A. Khan

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PREFACE

Plant kingdom is a vast storehouse of substances for food, medicine, timber, fibre and other economically important materials. With long experience mankind has learned that certain plant contain chemical substances which possess curative properties and therefore, could be employed for the treatment of various ailments. It is thus that plant drugs have attracted the attention of mankind for a long time. Until the begining of the great era of natural products chemistry in mid-ninteenth century, these medicinally important chemical constituents remained ill defined. With the advent of modern methods of separation and sophisticated spectroscopic techniques, the stereostructure and the biogenetic interrelationship along with their synthesis was worked out.

There is ample evidence of plant products which have proved valuable in the treatment of different diseases. For example, Rauwolfia serpentina had yielded hypotensive alkaloid reserpine, rescinnamine and deserpidine, Cinchona species had given antimalarial agents cinchonine and quinine, Digitalis purpurea had afforded digitoxin and digoxin,both possessing cardiotonic activity and Vinca rosea had furnished vinblastine as the anticancer agent.

The present dissertation incorporates the results of extensive chemical investigation of Pueraria tuberosa

which possess antiimplantation and early abortifacient activity. The 100% post-coital contraceptive activity at a dose level of 150 mg/kg in rats and hamsters model had been confirmed earlier at our institute.

P.tuberosa had afforded different class of compounds including the pterocarponoids, isoflavonoids and their derivatives. A number of isoflavones, coumestans etc. are known to elicit estrogenic effects. With these observations, the detailed chemical investigation of P. tuberosa was taken up.

The first chapter covers the survey on sources, structure and biological properties of chemical constituents of different Pueraria species distributed world-wide, while the second chapter concentrates on the recent findings on P.tuberosa which includes phytosterols, oleanene series of triterpenes, simple pterocarpans, isoflavonoid derivatives and other structurally interesting compounds.

CHAPTER I

Pueraria Species: An Overview

I. Pueraria species : An Overview

I.1 Introduction:

Pueraria[1] is a genus belonging to family Leguminosae (sub family. Papilionaceae) and distributed throughout the tropical and subtropical regions of the world which include countries; like, India, Burma, Thailand, Taiwan, Korea, a part of China and Japan. The genus is considered to be of immense medicinal value since immemorial time and described in the ancient medical literature of the oriental world.

In India, P.tuberosa[2,3] is mentioned in Ayurvedic system of medicine for several therapeutic uses, like, antipyretic, antidiuretic, antiaphrodisiac and galactogogue purposes[3]. Pueraria tubers are used in fever, rheumatism and as an emetic tonic by tribes. It is highly effective in dysmenorrhea, dysfunctional uterine bleeding and menopausal syndrome[4].

P.tuberosa is found in Western Himalayas, Kumaon hills, Sikkim, lower hills of Punjab and Mount Abu regions. It is also found in hilly tracts of Bengal and Nilgiri hills of Southern India[5].

The genus Pueraria (P.thunbergiana, P.pseudohirsuta and P.thomsonii) is also known as an important medicament

in Chinese system of traditional medicine. It is reported to possess antispasmodic activity and capable of stimulating blood circulation.

On the world map P.thunbergiana, P.pseudohirsuta and P.mirifica are distributed in southeast Asian countries while P.thomsonii, P.montana and P.tonkiensis are found in northeast regions of the world which includes Taiwan, Japan and northern province of China.

In view of the immense pharmacological importance attributed to Pueraria species world over, it had been undertaken for detailed chemical examination by many research groups in the world.

A brief summary which follows, is to review the uptodate work on sources, structure and biological properties of genus Pueraria which contains mainly the isoflavonoids and their glycosides.

Following Pueraria species had been chemically investigated:

i) Pueraria thunbergiana Benth (Syn. lobata)

ii) P.tuberosa (Roxb. ex. Willd) D.C.

iii) P.mirifica

iv) P.montana (Merril)

v) P.pseudohirsuta (Tang et Wang)

vi) P.tonkiensis (Gagn.)

vii) P.thomsonii (Benth.)

The P.thunbergiana and P.tuberosa are among the most extensively studied species followed by P.mirifica while P.edulis and P.phaseoloides are still chemically unexplored.

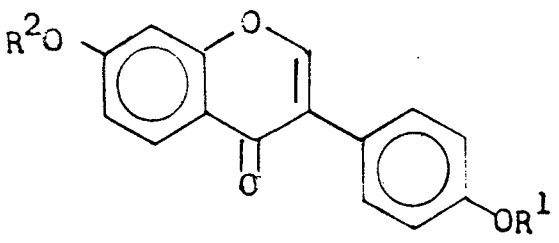
I.2 Chemical Constituents of P.thunbergiana:

Isoflavonoids are among the major identified constituents of P.thunbergiana whose vast majority occurs as free aglycones rather than combined with glucose or some other mono or disaccharide in the form of glycosides. This species represents the major share of isoflavonoids encountered so far in the genus.

Daidzein (1) (4',7-dihydroxyisoflavone) is the most abundant isoflavone identified in flowers and rhizomes of P.thunbergiana[6,7].

The occurance of rare isoflavone formononetin or biochanin-B (2) (4'-methoxy-7-hydroxyisoflavone) from the ether extract of the flowers and rhizomes of P. thunbergiana[8], and non-leguminous plant wood of Virola multinervia (Myristicaceae) had been established earlier[9].

Genistein (3) (4',5,7-trihydroxyisoflavone) was first reported from the leaves of Genista germanica[10] and its presence in P.thunbergiana[6] was established later in 1973. The chemical literature survey revealed that the compound (3) is indexed under three different



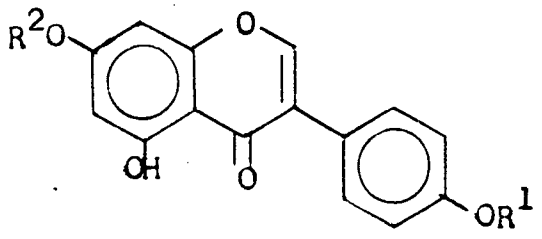
(1) R^1=H, R^2=H

(2) R^1=Me, R^2=H

(8) R^1=H, R^2=Glu

(9) R^1=Me, R^2=Glu

(17) R^1=Glu, R^2=Glu

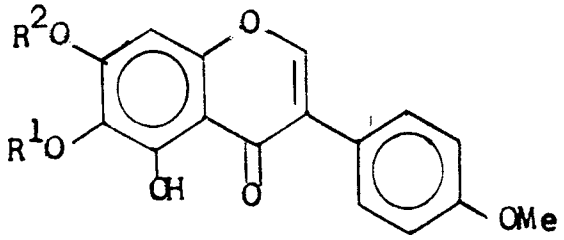


(3) R^1=H, R^2=H

(4) R^1=Me, R^2=H

(10) R^1=H, R^2=Glu

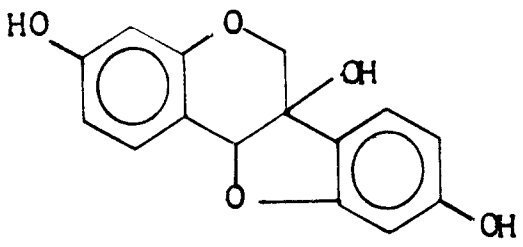
(11) R^1=Me, R^2=Glu



(5) R^1=Me, R^2=H

(12) R^1=Me, R^2=Glu

(13) R^1=Me, R^2=Xylosyl (1->6)glu



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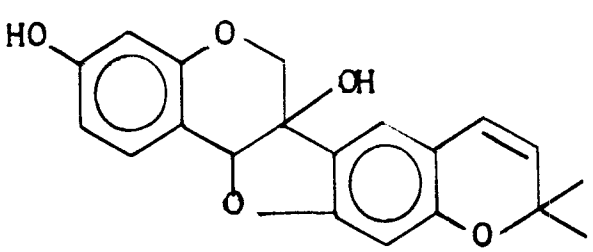


(7)

names, i.e., genistein, prunetol and sophoricol; owing to its presence in non-leguminous plant wood of Prunus aequinoctialis[11] (Rosaceae), and fruits of Sophora japonica[12] respectively. Another compound identified as hydroxy derivative of prunetol, named, pratensol or biochanin-A (4) (5,7 -dihydroxy-4'-methoxyisoflavone) was isolated and characterised from the flower petals of the plant[6,8].

Irisolidone (5) (4',6-dimethoxy-5,7-dihydroxy-isoflavone) was extracted from the rhizomes of Iris kashmiricana (Iridaceae)[13], and its presence in P. thunbergiana[6] was established in 1973.

The pioneering contribution came from the laboratory of J.L.Ingham et al. who established the presence of pterocarponoids in Pueraria species. The ptercarponoids are considered as 'phytoalexins' produced as a consequence of invasion and attempted colonisation of the plant organ or tissue cells by the pathogenic microorganisms, which are most often a fungi or bacteria[14]. The pterocarponoids are mostly part of the defensive mechanism of the leguminous plant species[15,16]. The compounds with isoprenoid substituents are reported to be extremely toxic to the saprophytic and pathogenic fungi[17,18] and this remarkable activity had created an interest in the isolation

and characterisation of the pterocarponoids. Among the first pterocarponoid isolated was glycinol (6), a 6-oxypterapan from the seeds of Glycine max[19]. The compound lacks isoprenoid system in its molecular framework. Its presence in P.thunbergiana was reported by J.L.Ingham. The second pterocarponoid, a 6-oxy-pterocarpan; tuberosin (7), with the cyclised isoprenoid system in the form of linearly fused 2,2-dimethylchromene ring as part of their structure was established by Joshi and Kamat (1973) from the chloroform extract of the tubers of P.tuberosa[20] and later its presence was confirmed in P.thunbergiana[21].

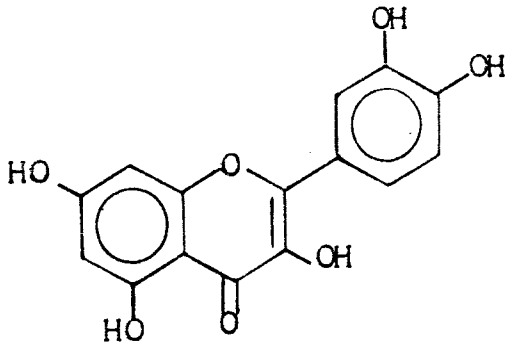
The polar fraction of P.thunbergiana which contained major isoflavonoid glycosides incorporating glucose and/or other mono or disaccharide sugars had been studied extensively. Although the vast majority of the compounds occur as O-glycosides, but C-glyco-sylation is by no means an uncommon step in the molecular structures of the compounds found in these plants.

Among the major O-glycosides of isoflavone are daidzein-7-O-glucoside (daidzin)[22] (8), formononetin-7-O-glucoside (ononin)[8] (9), genistein-7-O-glucoside (genistin)[8] (10), biochanin-A-7-O-glucoside (astroside)[8] (11), irisolidone-7-O-glucoside (kakkalidone)[23] (12) and irisolidone-7-O-xylosylglucoside (kakkalide)[24] (13).

The occurance of other flavonoid compounds was detected with the isolation of quercetin (14) as flavone component, in addition to the essential oils from the fresh flowers of P.thunbergiana by Kurihara and Kikuchi[25].

I.3 Puerariae Radix, the dried roots of Pueraria lobata (Syn. thunbergiana) of Japanese origin is an important crude drug in China, Korea, Taiwan and Japan. It had been used for treatment of crapulence and to relieve the severe headaches. It had also been used as diaphoretic, antitussive and antidiarroheal agent[26]. A number of isoflavone derivatives and other structurally related compounds are reported from this plant. Two novel glycosides, 5-[[6-O-(6-deoxy-α-L-rhamnopyranosyl)-β-D-glucopyranosyl]oxy]-5,6-dihydro-9-hydroxy-4-(4-hydroxyphenyl)-2H-1-benzoxocin-2-one (pueroside-A) (15) and 5-(β-D-pyranosyloxy)-4-[4-(β-D-glucopyranosyloxy) phenyl]-5,6-dihydro-9-methoxy-2H-1-benzoxocin-2-one (pueroside-B) (16) were isolated and characterised by T.Nohara et al. in 1985[27].

Among the isoflavone glycosides the 7,4'-di-O-glucoside of daidzein (17), 8-C-glucoside of daidzein (18), PG-1 (19) and methoxy derivative of PG-1, coded as PG-2 (20), are partially characterised from the callus tissue of P.lobata[26].



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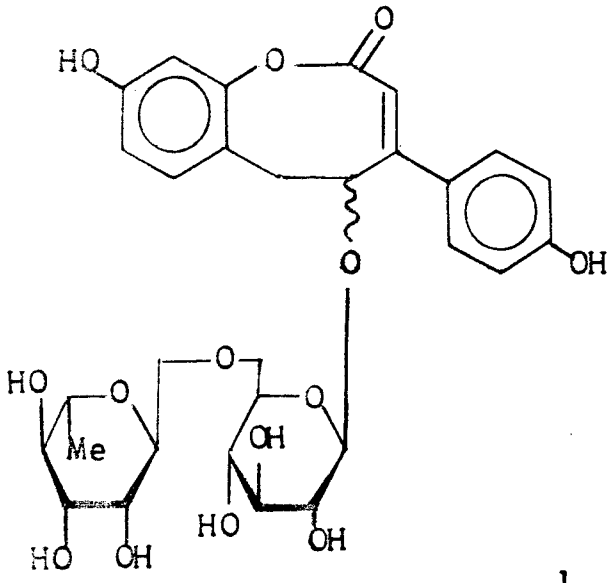

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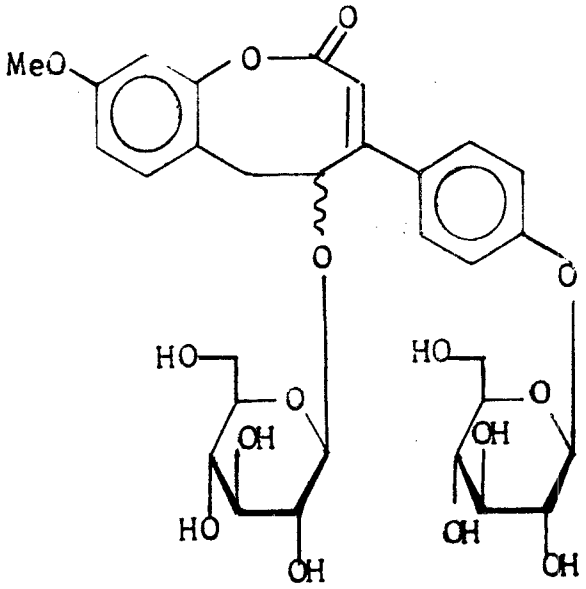

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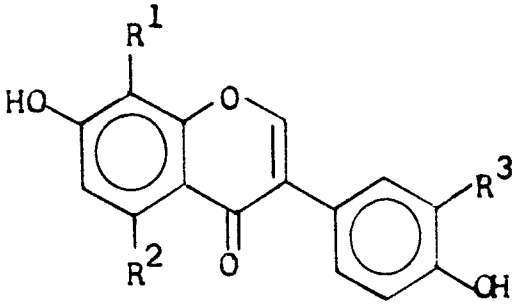


(18) R^1=Glu, R^2=H, R^3=H

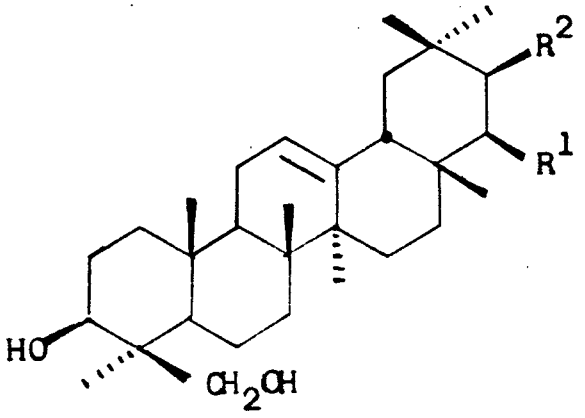
(21) R^1=Glu, R^2=H, R^3=OH

(22) R^1=Glu, R^2= H, R^3=OMe

The presence of irisolidone-7-O-glucoside[23] (12), hydroxy and methoxy derivatives of puerarin, characterised as 3'-hydroxy puerarin (21) and 3'-methoxy puerarin (22) are worth mentioning compounds from this plant[28]. Irisolidone-7-O-xylosylglucoside (13) and, puerarin xyloside (23) had also been reported by Japanese workers[29] in 1960.

Recently, two more isoflavone glycosides have been isolated by J.Kinjo et al. (1987) and characterised as 8-C-apiosyl (1->6) glucoside of daidzein (24) and 8-C-apiosyl (1 -> 6) glucoside of genistein (25) from the roots of the plant[30]. Among the pterocarponoids, the presence of 6-oxypterocarpan, glycinol (6) and tuberosin (7) was established along with the coumestan derivative, puerarol (26), and daidzein (1), formononetin (2), genistein (3) as free isoflavone aglycones[26] in 1975.

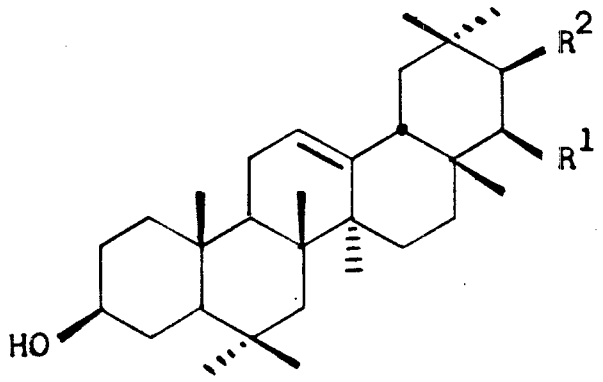
Puerariae Radix, in addition to a large number of isoflavonoids, had exceptionally yielded triterpene saponins from the polar fractions. No report had previously appeared with respect to the occurance of triterpenoid ingradients in genus Pueraria. However, in view of their presence in leguminous plants, such as soybeans, Glycine max[31] and Astragalus membranaceus[32], it seems quite natural to encounter the presence of triterpenes and their glycosides. The triterpene sapogenols include soyasapogenol-A (27), soyasapogenol-B



(27) R^1=OH, R^2=OH

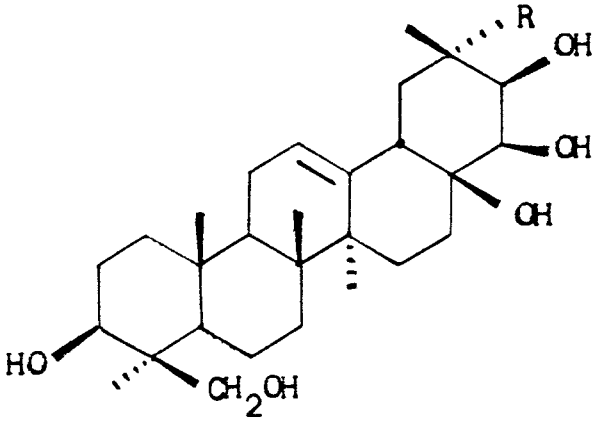
(28) R^1=OH, R^2=H

(32) R^1=H, R^2=OH



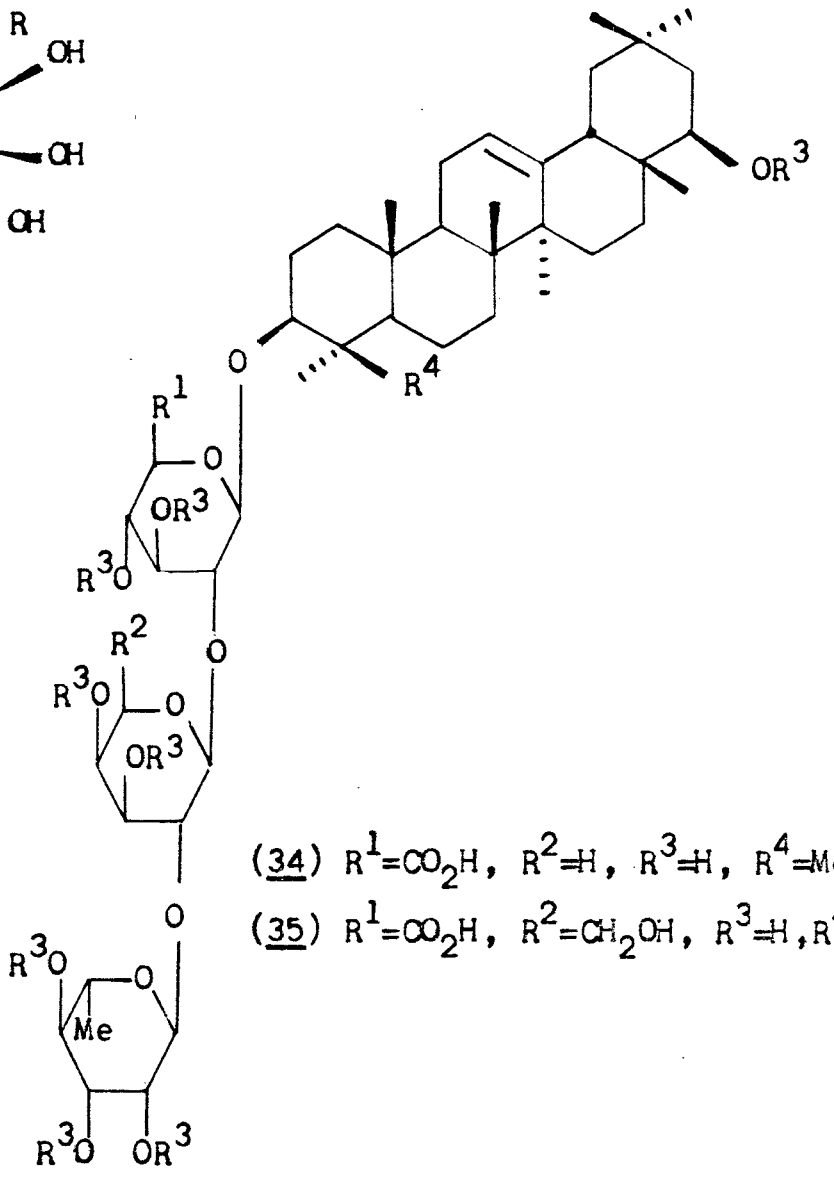
(29) R^1=OH, R^2=H

(30) R^1=OH, R^2=OH



(31) R=CH_2OH

(33) R=CO_2Me



(34) R^1=CO_2H, R^2=H, R^3=H, R^4=Me

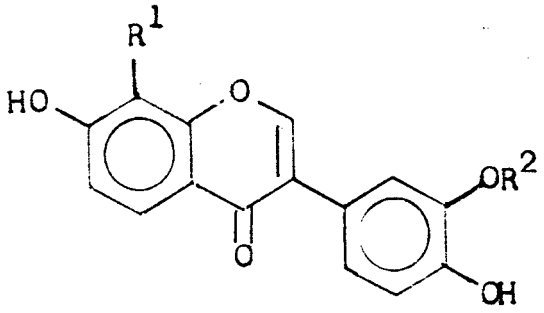
(35) R^1=CO_2H, R^2=CH_2OH, R^3=H, R^4=Me

(28), sophoradiol (29), cantoniensistriol (30), kudzusapogenol-A (31), kudzusapogenol-C (32) and methylester of kudzusapogenol-B (33) as the major components from this plant[33]. The occurance of 3-O-[α--L-rhamnopyranosyl (1 -> 2)-α-L-arabinopyranosyl (1 -> 2)--β-D-glucopyranosyl]-sophoradiol (34) and kaikosaponin-III (35) had been identified as saponins by T.Nohara et al.[34].

I.4 Chemical Constituents of P.tuberosa:

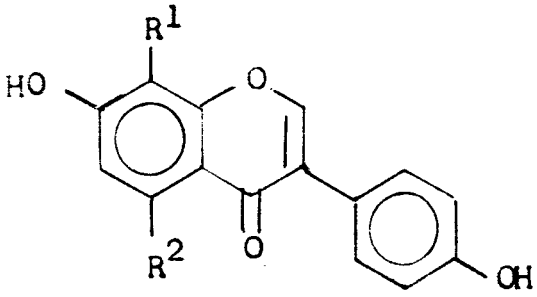
The species is native of India and found almost throughout the country upto the altitude of 1,200 metres. It had been used by tribes in various disorders and confirmed to possess estrogenic as well as progestogenic activity. It is reported to contain a number of isoflavonoids and their glycosides. The presence of daidzein (1)[22], dihydrodaidzein (36)[36], kakkatin (37)[35] and a prenylated compound characterised as neobavaisoflavone (38) had been established[36].

Inspite of the preponderance of isoflavonoid, P.tuberosa contains a considerable number of pterocarponoids and triterpene glycosides. The interesting piece of work on these class of compounds in P.tuberosa was reported by R.S.Kapil et al., which enlists the occurance of anhydrotuberosin (39), 3-O-methylanhydrotuberosin (40) from pterocarpenes[37], while tuberostan (41) and a



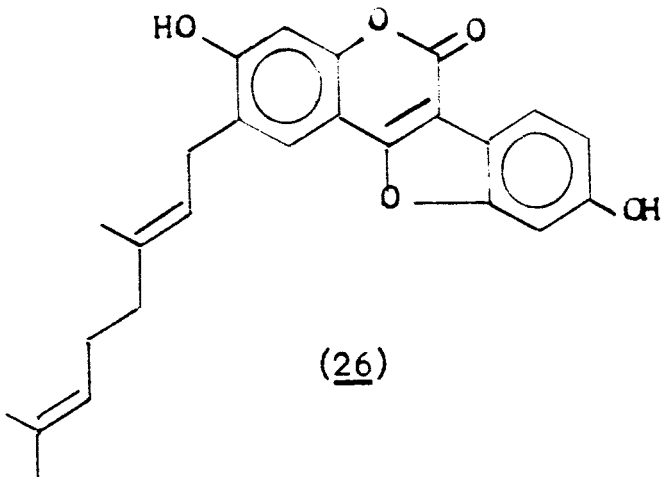
(19) R^1=Sugar, R^2=H

(20) R^1=Sugar, R^2=Me



(24) R^1=Apiosyl(1->6)glu, R^2=H

(25) R^1=Apiosyl(1->6)glu, R^2=OH



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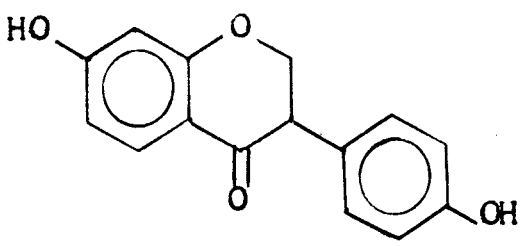

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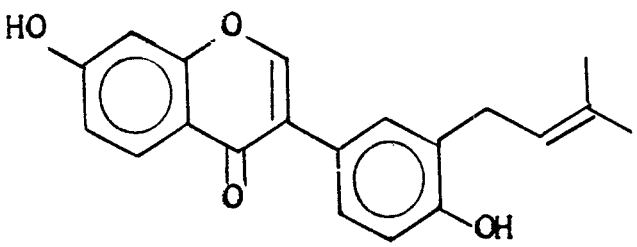

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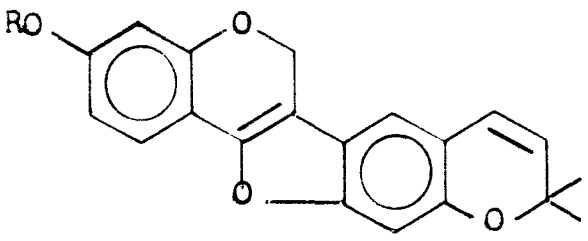
structurally interesting fungal metabolite, 1a-hydroxy--tuberosone (42) are among the representatives of coumestan and pterocarpanone class of compounds respectively[38]. The tuberosin (7) is the sole example from 6a-oxypterocarpan. The characteristic feature in all the pterocarponoids isolated so far from P.tuberosa, is the presence of a linearly fused 2,2-dimethylchromene ring as part of their structures[36-38].

The polar fractions of P.tuberosa yielded 4',6''-di-O-acetylpuerarin (43)[22] and daidzein-8-C-glucoside (18)[29,39]. The later is the foremost representative of C-glycoside isolated from any natural source.

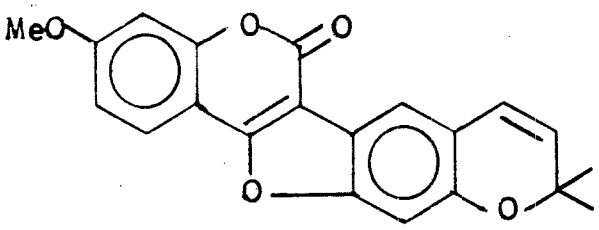
I.5 Chemical Constituents of P.mirifica:

The species is encountered in Burma and Thailand. It had been used for the treatment of harmonal disorders by Buddhist monks for many decades. J.C.Cain (1965) reported the isolation and structure elucidation of an estrogenic component, miroestrol (44) from the tubers of P.mirifica with the help of X-ray crystallographic technique, in addition to the other physico-chemical method of structure determination[40]. Recently, the presence of coumestrol (45),and a prenylated isoflavone kwakhurin (46) [7,2',4'-trihydroxy-5'-methoxy-6'-(3,3-dimethylallyl)isoflavone] had also been reported[41].



(39) R=H

(40) R=Me



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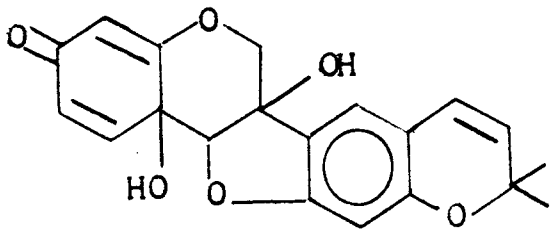

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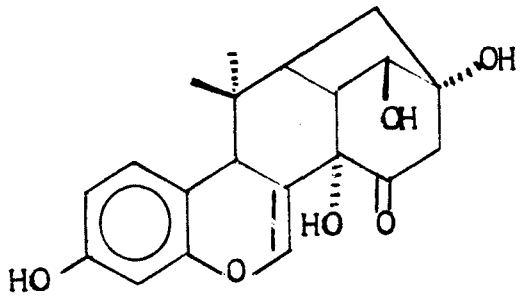

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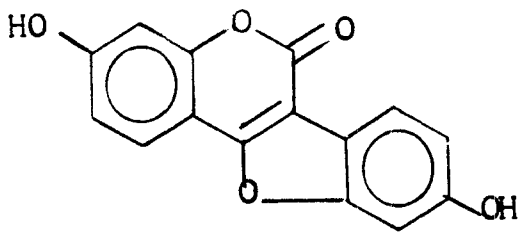

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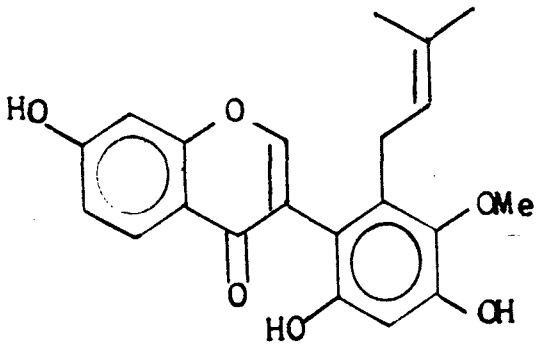


(46)

J.L.Ingham *et al.*, had reported another puerarin derivative identified as puerarin-6"-O-β-apiofuranoside (47) from the n-butanol fraction of *P.mirifica*[42].

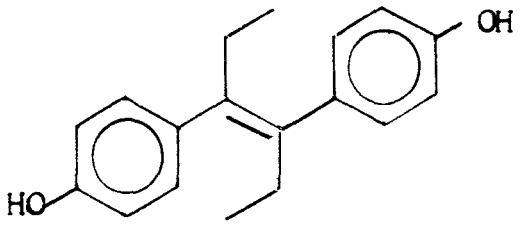
I.6 The least examined species in this series are *P.montana* of Taiwanese origin, *P.pseudohirsuta*, *P. tonkiensis* and *P.thomsonii* from the northeastern region of Asia. These species had been poorly investigated for their chemical constituents. Among the identified products, irisolidone-7-O-glucoside (12),and tectorigenin-7-O-glucoside (13) are some of the major isoflavone glycoside detected by the physical methods[23].

I.7 Biological Activity and Uses of Compounds from *Pueraria* Species:

The chemical constituents isolated from *Pueraria* species display an exceedingly diverse range of biological properties depending upon the structures of certain compounds. The presence of considerable number of pterocarponoids and other isoflavones with isoprenoid substituents in it, works as phytoalexin to the plant itself and fight against the invasion and colonisation of the pathogenic microorganisms[43]. These defensive components are distributed in roots, barks, stems, leaves and flowers.

A few isoflavonoid are nematistic and anti-bacterial to gram-positive strains of bacteria. Pterocarponoids and a range of isoflavone aglycones which includes daidzein (1), formononetin (2), genistein (3) and biochanin-A (4) and their glycosides have been found to inhibit the root and apex growth of the plants. Thus the anti-auxin and anti-gibberalin activity associated with these compounds may perhaps function as natural growth-regulators[44].

Several compounds that mimic the activity of animal estrogens have been identified as either estrogens or pro-estrogens i.e. compounds which may change into more active substances inside the body of animals. The most important among these are daidzein (1), formononetin (2), genistein (3), biochanin-A (4) and coumestrol (45). The daidzein (1), genistein (3) and biochanin-A (4) are weaker in action, whereas the formononetin (2) and coumestrol (45) are considerably more potent, but less than the ovarian estrodiol (48) and synthetic estrogen diethylstilboestrol (49), to which they bear a profound structural resemblance (Chart 1). The difference in activity between these compounds is related to their mode of action inside the body. The coumestrol (45) appears to be more effective as it is less amenable to degradation[45,46].



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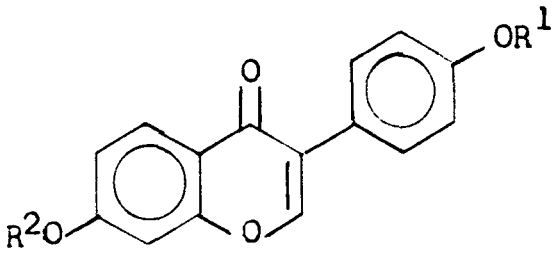
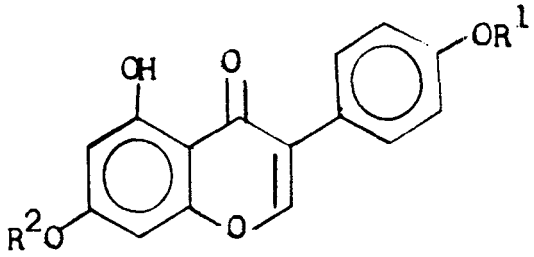


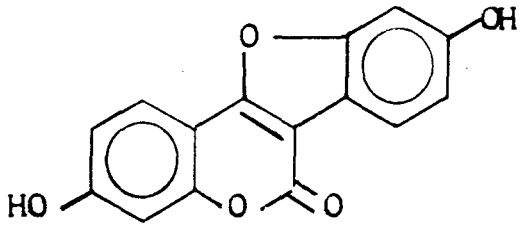
(1) R^1=H, R^2=H

(2) R^1=Me, R^2=OH



(3) R^1=H, R^2=H

(4) R^1=Me, R^2=H



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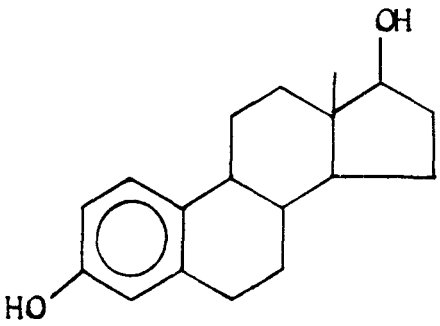

(48)

Chart 1

Recently, a group of Chinese workers have reported antihypertensive activity in puerarin (18)[47]. It was formerly known to dilate coronary artery, decrease blood pressure, heart rate and vascular resistance[48].

Some additional minor biological properties ascribed for these natural products are summarised in Table 1.

Table 1: Minor Properties of Compounds Isolated from *Pueraria* Species

Property/Use	Compounds	References
1. Anti-haemolytic activity	Daidzein Genistein Genistin	49,50,51
2. Anti-spasmodic activity	Daidzein	52
3. Anti-oxidative activity	Genistein	50,51
4. Anti-microbial and anti-fungal activity	Tuberosin	53
5. Anti-bacterial and anti-tubercular activity	Tuberosin	20
6. Anti-implantation activity	Tuberosin	53
7. Anti-ulcer activity	Genistein Biochanin-B	54
8. Diuretic property	Tectoridin	55
9. Enzyme inhibition:		
a) Catechol-O-methyl transferases	Genistein	56
b) DOPA decarboxylase	Genistein	57,58
c) Dopamine β-hydroxylase	Genistein	57
d) Histidine decarboxylase	Genistein	57
e) Lipase	Daidzein Genistein Daidzin	59
f) Hypocholesterolemic activity	Biochanin-A Biochanin-B	60
g) Hypolipidemic activity	Biochanin-A Biochanin-B	61,62

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CHAPTER II

Chemical Investigation of *Pueraria tuberosa*

II. Chemical Investigation of *Pueraria tuberosa*:

II.1 Introduction:

Pueraria tuberosa[1,2] (Roxb. ex. Willd) D.C. (Leguminosae) is commonly known as Vidarikand in Vernacular language. It is also called as 'Indian Kudzu' since it resembles the common 'Kudzu' in many features. The tubers of the plant are huge, 30-35 cm broad and 40-60 cm long, weighing upto 40 kg. They are used for extraction of starch and occassionally eaten raw. It had been traditionally used for fertility regulation by nomadic tribes in Jammu and Kashmir regions of the country. The preliminary pharmacological trials of the crude ethanolic extract of the plant tubers has confirmed the post-coital contraceptive activity[3].

The study of the extracts of *P.tuberosa* showed the vaginal cornification in ovariectomised adult rats and uterotrophic activity in immature rats which inturn revealed the harmonal profile of the plant at a dose level of 200 mg/kg[4]. The estrogenic activity was further confirmed by increased uterine, cervical and vaginal weights, and enhanced glycogen, protein and acid and alkaline phosphatase content of these organs in ovariect-omised rats at 125 mg/kg dose level. The changes

in estrogen primed uterine endometrium of immature rabbits at 50-200 mg/kg dose level showed the progestogenic activity of the crude drug. From these studies it was evident that a naturally occurring mixture, showing both the estrogenic and progestogenic activities in the same extract may be responsible for the contraceptive action of this plant.

II.2 Present Work:

In view of the post-coital contraceptive efficacy of the P.tuberosa, it was undertaken for detailed chemical investigation to isolate and characterise the active constituent(s). The 95% ethanolic extract was found to possess 100% post-coital contraceptive activity in rats and hamsters model at a dose level of 150 mg/kg. On further fractionation the activity got concentrated in chloroform and n-butanol fractions, which on repeated column and flash chromatography led to the isolation of several compounds as listed in Table 1.

Table 1: Substances isolated from P.tuberosa

Sl. No.	Substance	Molecular formula	m.p. (°C)	Identified as
1.	Substance A	$C_{16}H_{12}O_4$	133	Isomedicarpene*
2.	Substance B	$C_{29}H_{50}O$	136	β-Sitosterol.
3.	Substance C	$C_{29}H_{50}O$	156	Stigmastan-3-one**
4.	Substance D	$C_{30}H_{50}O$	290	β-Amyrin**
5.	Substance E	$C_{20}H_{16}O_5$	-	Pueranone*
6.	Substance F	$C_{30}H_{50}O_3$	256	Soyasapogenol-B**
7.	Substance G	$C_{30}H_{48}O_3$	306	Oleanolic acid**
8.	Substance H	$C_{30}H_{48}O$	168	Lupen-3-one**
9.	Substance I	$C_7H_6O_3$	216	p-Hydroxybenzoic** acid
10.	Substance J	$C_6H_6O_3$	164	Maltol**
11.	Substance K	$C_{15}H_{10}O_5$	-	Hydroxydaidzein**
12.	Substance L	$C_{21}H_{20}O_9$	236	Daidzin.

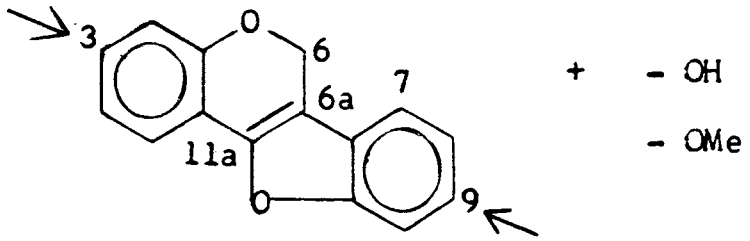
* New Natural Products

**New to the plant.

Substance A, m.p.133°.

It was obtained as pale yellow crystals on crystallisation with hexane-chloroform. Its UV spectrum showed absorption maxima at 280, 306 and 340 nm which clearly indicated the presence of pterocarpene nucleus[5]. The mass spectrum of compound exhibited the molecular ion peak at m/z 268 corresponding to the molecular formulae $C_{16}H_{12}O_4$. The IR spectrum showed the presence of hydroxy group at 3400 cm^{-1} while it did not exhibit any absorption band in the carbonyl region which ruled out the possibility of coumestan type of the nucleus. The interlocking evidence provided by the PMR spectrum of the compound showed the presence of a two protons singlet at δ 5.40 assignable to the characteristic pterocarpene C-6 methylene group[6]. On the basis of these data it can be inferred that, in the substance-A, all the four oxygen atoms, accounted by molecular formulae, were present either as hydroxy group or as a part of the ether functionality. The substance-B on acetylation with acetic anhydride and pyridine formed a monoacetate (2) which showed molecular ion peak at m/z 310 accounting for the increase of one acetyl group in the parent compound. Moreover, a shift in the UV spectrum at 305, 350 and 355 nm on the addition of alkali suggested the presence of one oxygen atom as part of the phenolic hydroxy group[7]. The second

oxygen atom was accounted by the observation of PMR spectrum which showed a methoxy group resonating at δ 3.70 in the form of a singlet integrating for three protons. The rest of the two oxygen atoms were accounted for the parent benzofuran moiety of the substance-A which is a part of the constitution of the molecule. These data led to the confirmation of a pterocarpene nucleus with one hydroxy and one methoxy group as the substituents.



The biogenetic consideration[8] of the 3,9-dioxygenation pattern of pterocarpene moiety helped to assign the position of these two groups, i.e., methoxy at C-3, and hydroxyl group at C-9 position, which is also in accordance with the previously isolated constituents of P.tuberosa[9]. These observations ultimately led to the 3-methoxy-9-hydroxypterocarpene (isomedicarpene) structure (1) for the substance-A.

Substance B, m.p.136° .

It was obtained as colourless flakes from methanol. It gave positive colouration with tetranitromethane and

showed deep bluegreen colour with Libermann-Burchard test; indicating it to be an unsaturated sterol[10]. In its mass spectrum it showed a molecular ion peak at m/z 414 which was in complete accordance with the assigned molecular formulae $C_{29}H_{50}O$. The IR spectrum showed strong absorption bands at 3650 (br) and 1040 cm^{-1} for hydroxy group[11]. The absorption bands at 1640 cm^{-1}, and a pair of absorption bands at 835 and 800 cm^{-1} were accounted for the presence of trisubstituted double bond (>C=CH) in the compound[12]. The UV spectrum showed absorption maxima at 204 nm which was typical of the steroidal nature of the compound[13]. The PMR spectrum of the compound exhibited a bunch of singlets ranging from 0.62-0.96 for six methyl groups showing an integration for eighteen protons, a one proton multiplet at δ 3.60 for the carbinolic proton (>CH-OH) attached to C-3 position in accordance with the biogenetic origin[14], and one proton multiplet at δ 5.44 for the presence of one methine proton (>C=CH) at C-5. The PMR spectrum also showed a broad hump at δ 1.20-1.84 for rest of the twenty two protons of methylene kind in the molecule.

These foregoing data led to the basic skeleton of sterol with one hydroxy group and a trisubstituted double bond placed at C-3 and C-5 positions respectively. It was later confirmed from the evidences followed from

the mass spectrum which was very informative. The presence of intense ion peaks at m/z 273 (M^+ -side chain), 255 (M^+ -side chain-H_2O), 231 [M^+ -side chain-42(ring D fragment)], and 213 (231-H_2O) were characteristic of stigmastane skeleton. The principal peak at m/z 213 led to the placement of $C_{10}H_{21}$ side chain at C-17 position in ring D of the molecule and this eliminated the possibility of unsaturation in the side chain. All these chemical and spectroscopic properties accounted well with the β-sitosterol structure (3) for substance-B. It was later finally confirmed with an authentic sample in all respect (m.p., m.m.p., co-T.L.C., co-IR and $[\alpha]_D$) and unequivocally established its identity.

Substance C, m.p.156°.

The substance-C was crystallised from dichloromethane as white crystals. It was assigned the molecular formulae $C_{29}H_{50}O$ on the basis of its mass spectrum which showed the molecular ion peak at m/z 414. It gave positive Libermann-Burchard test turning deepred colouration to purple and ultimately to green, indicating it to be a steroid[10]. The UV spectrum showed absorption band for the presence of a carbonyl group and the observation of IR spectrum indicated the presence of ketone moiety by appearance of strong absorption band at 1710 cm^{-1}

while it did not show any absorption band in hydroxy group region[11]. Thus the tentative structure of the compound was followed as a steroid with carbonyl group incorporated in it. The PMR spectrum which exhibited a bunch of singlets ranging from δ 0.70-1.10 giving an integration for eighteen protons accounted for six methyl group, and a four protons multiplet at δ 2.20-2.50, assignable to two methylene groups (-$\underline{CH}_2$-CO-$\underline{CH}_2$-) adjacent to the carbonyl group which was placed at C-3; causing a downfield shift in the chemical shift positions of methylene protons. The PMR spectrum further evidenced the presence of methylene protons at δ 1.2-1.95 for twenty protons in all and this ruled out the possibility for the presence of any other functional group in the molecule. The mass spectrum which showed prominent ion peaks at $\underline{m}/\underline{z}$ 399 (M^+-Me), 273 (M^+-side chain), 271, 229 and 203, followed the course of fragmentation of a stigmastane skeleton[15,16]. The mass spectrum further showed the fragmentation pattern for the acyclic side chain incorporated into the molecule[17]. These data led to conclude substance-C as stigmastan-3-one (4), which was later confirmed by comparison with an authentic sample (m.p., m.m.p., co-T.L.C., co-IR and $[\alpha]_D$) of stigmastan-3-one.

Substance D, m.p.290°.

It was crystallised from methanol affording white crystals and analysed for $C_{30}H_{50}O$ on the basis of its mass spectrum which exhibited the molecular ion peak at m/z 426. It responded to the colour reactions of Libermann-Burchard test for triterpenoids turning from a red colour to deep blue[18]. The observation of IR spectrum revealed the presence of hydroxy group and trisubstituted double bond[11,12] which showed absorption bands at 3450 and 1045 (OH); 1640, 840 and 780 cm^{-1} for trisubstituted double bond (>C=CH). The complimentary evidences were followed from the mass spectrum which showed the presence of prominent ion peak at m/z 218, formed by the retro Diels-Alder fragmentation of the molecule. The mass spectrum followed the fragmentation pathway for typical oleanene series of triterpene[19]. The conversion of substance-D to its monoacetate derivative (M^+ m/z 468) confirmed the presence of one hydroxy group which was tentatively placed at C-3 in accordance with the biogenetic origin and later confirmed by the observation of prominent ion peak at m/z 218, formed by the loss of 208 mass units from the molecular ion. The retro Diels-Alder fragments also helped in to assign the C-12 position to trisubstituted double bond (>C=CH) as depicted in Figure 1.

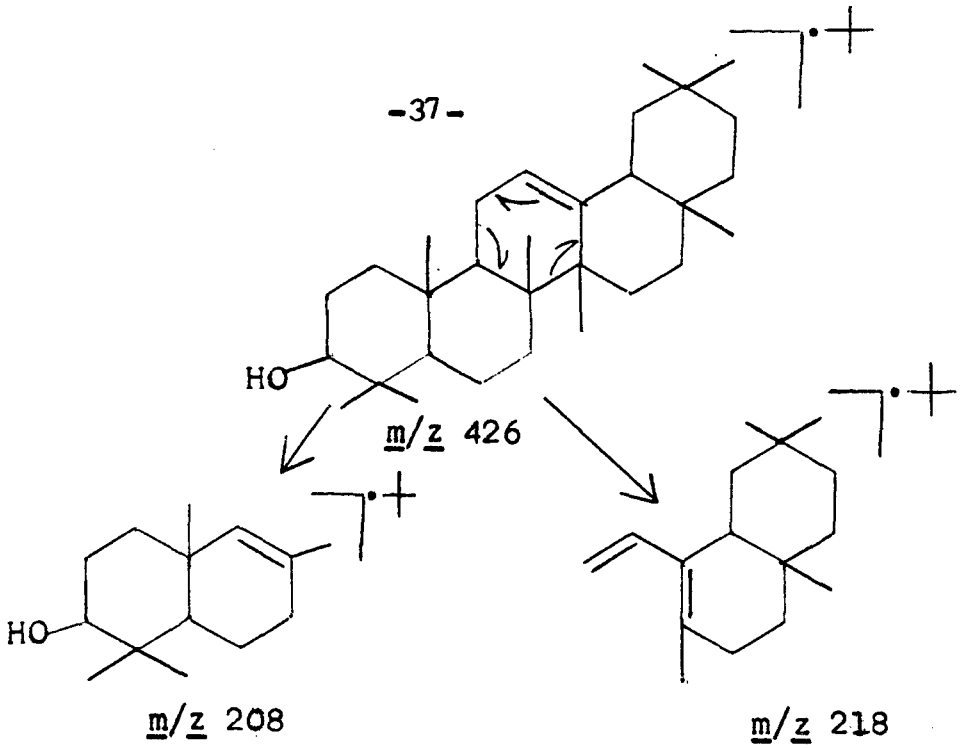


Figure 1

These informations were further substantiated by the PMR spectrum which showed a group of singlets for eight methyl groups of tertiary nature in the molecule ranging from δ 0.75-1.20 showing an integration for twenty four protons. The PMR spectrum also revealed the presence of a multiplet at δ 3.30 for carbinolic proton (>C<u>H</u>-OH), and the presence of trisubstituted double bond at C-12 was indicated by the appearance of one proton multiplet at δ 5.22 for one methine proton (>C=C<u>H</u>). The PMR spectrum also showed a broad hump at δ 1.7-2.1 for twenty protons of methylene type. These foregoing data along with the mass spectrum fragmentation pattern led to the 3β-hydroxy-olean-12-ene structure for substance-D. It was later confirmed by the direct comparison with the authentic material (co-T.L.C., co-IR, m.m.p. and $[\alpha]_D$). in all respects.

Substance E:

It was obtained as reddish oily material and the elemental composition was determined to be $C_{20}H_{16}O_5$ on the basis of mass spectrum which showed the molecular ion peak at m/z 336. It gave a brown colour with $FeCl_3$[20] but showed no colouration with the Shinoda test[21]. In its IR spectrum it exhibited strong absorption bands at 3400 cm^{-1} for hydroxy group and another absorption band at 1660 cm^{-1} was accounted for the presence of a conjugated carbonyl group in the molecule. The UV spectrum showed absorption maxima at 230 and 305 nm clearly indicating the isoflavone nature of the compound[7]. An alkali induced shift in the UV spectrum indicated the presence of phenolic hydroxy group which showed absorptions at 235 and 325 nm. The confirmation for the presence of hydroxy group in the compound was drawn by the preparation of acetyl derivative (8). The appearance of molecular ion peak at m/z 420 in the mass spectrum of acetate derivative indicated the presence of two hydroxy groups in the molecule. The interlocking evidences were provided by the PMR spectrum (400 MHz) which clearly showed a characteristic C-2 isoflavone singlet at δ 8.25 and a one proton doublet in the aromatic region at δ 8.05 (J=8.5 Hz) for an aromatic proton ortho to the carbonyl group . The further observation of PMR spectrum showed

the corresponding coupled proton seen as a double doublet at δ 7.10 (J=8.5 Hz, 2.5 Hz) and another one proton doublet at δ 6.94 (J=2.5 Hz) which supported the contention of the presence of an ABX system of protons in the molecule.

The remarkable exhibition in the PMR spectrum which decided rest of the infra structure of the molecule was the appearance of a six protons singlet at δ 1.37 and an AB quartet for two protons at δ 5.50 (J=10.0 Hz) and at δ 6.30 (J=10.0 Hz) which was flanked by two aromatic singlets at δ 6.28 and δ 6.95, each integrating for one proton, had established the presence of a 2,2-dimethylchromene ring in the molecule[22]. This was further confirmed by the appearance of base peak at m/z 321 in the mass spectrum of the compound which is formed by the loss of 15 mass units from the molecular ion[23] as depicted in Figure 2. The linear disposition of the 2,2-dimethylchromene ring was decided by the observation of two aromatic singlets for C-2' and C-5' protons in the PMR spectrum of the compound. These foregoing data led to the tentative structure of the isoflavonoid with two hydroxyl and a dimethylchromene ring. A decision was made based on the biogenetic considerations[8] and comparison with the PMR spectral values of the previously isolated known isoflavone compounds from *P. tuberosa*[24]. This

unequivocally led to structure (7) for substance-E with a linearly fused 2,2-dimethylchromene ring and is in complete accordance with the physico-chemical data.

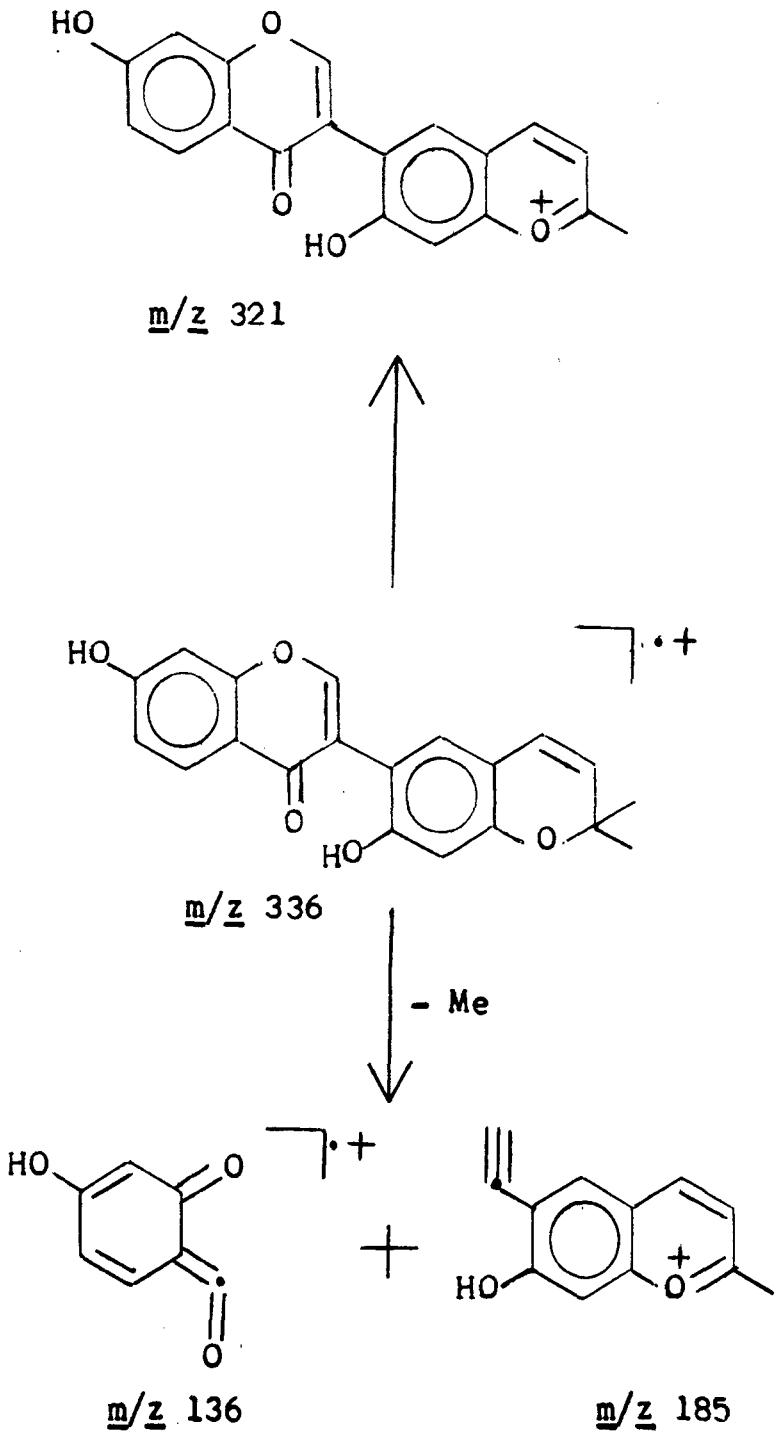
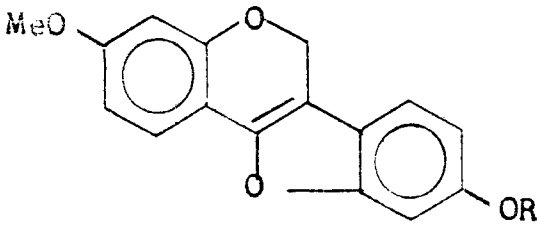


Figure 2

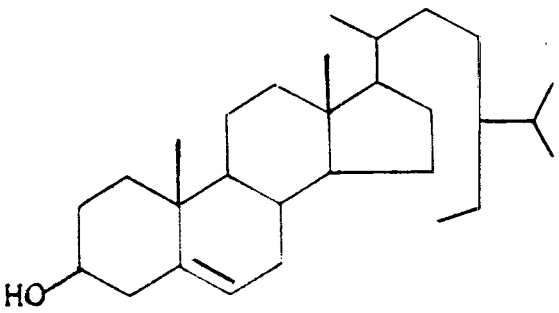
Substance F, m.p.256°.

Substance F was analysed for $C_{30}H_{50}O_3$ on the basis of its mass spectrum which showed a clear molecular ion peak at m/z 458. Its IR spectrum displayed strong absorption bands at 3450 and 1040 cm^{-1} for hydroxy group[11], and absorption bands at 1640, 835 and 820 cm^{-1} were indicative of the presence of trisubstituted (>C=CH) double bond[12] in the molecule. It responded positively to the Libermann-Burchard test for triterpenes[18] and showed characteristic mass spectral fragmentation pattern for oleanene series of triterpenes[19]. The appearance of one of the fragment ion peaks at m/z 234 formed by the retro Diels-Alder fragmentation confirmed this contention. The final proof for the structure establishment came with the help of PMR spectrum which displayed a bunch of signals at δ 0.90-1.14 for seven methyl groups, showing an integration for twenty one protons and signals for methylene protons spread over δ 1.46-1.94. The methine proton (>C=C<u>H</u>) at C-12 resonated at δ 5.28 as a multiplet. The signal which resonated at δ 3.26 and 2.82 as multiplet were assigned to the carbinolic protons (>C<u>H</u>-OH) at C-3 and C-22 respectively[25]. The another carbinolic proton which was located at C-24 appeared as multiplet at δ 4.16 and was found to be primary in nature.

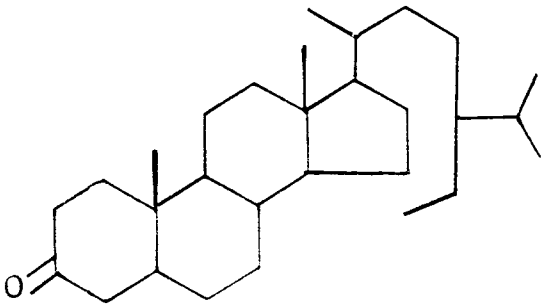


(1) R=H

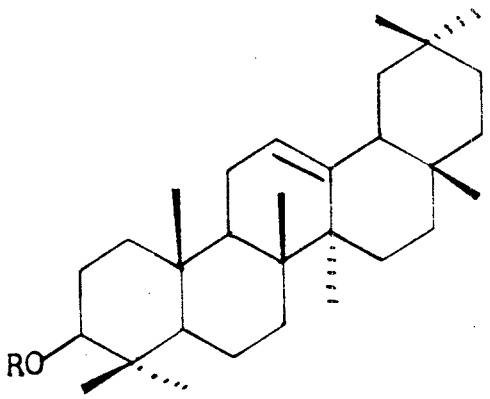
(2) R=Ac



(3)

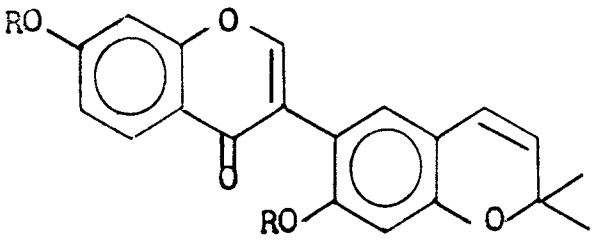


(4)



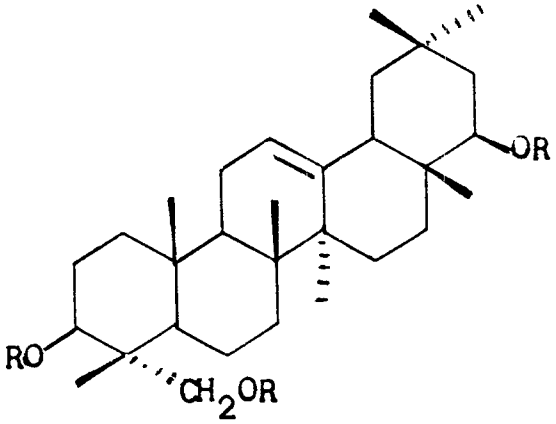
(5) R=H

(6) R=Ac



(7) R=H

(8) R=Ac



(9) R=H

(10) R=Ac

The mass spectrum of the compound showed prominent ion peaks at m/z 440, 234, 224 and 176 as depicted in Figure 3. Moreover, the acetate derivative (10) of the substance F showed the molecular ion peak at m/z 584 confirming the presence of three hydroxy groups in parent molecule. Lastly, the comparison with the authentic material in all respects (m.p., m.m.p., co-T.L.C., co-I.R. and $[\alpha]_D$) established the identity of substance F as soyasapogenol-B (3β, 22β, 24-trihydroxy olean-12-ene)[25] (9).

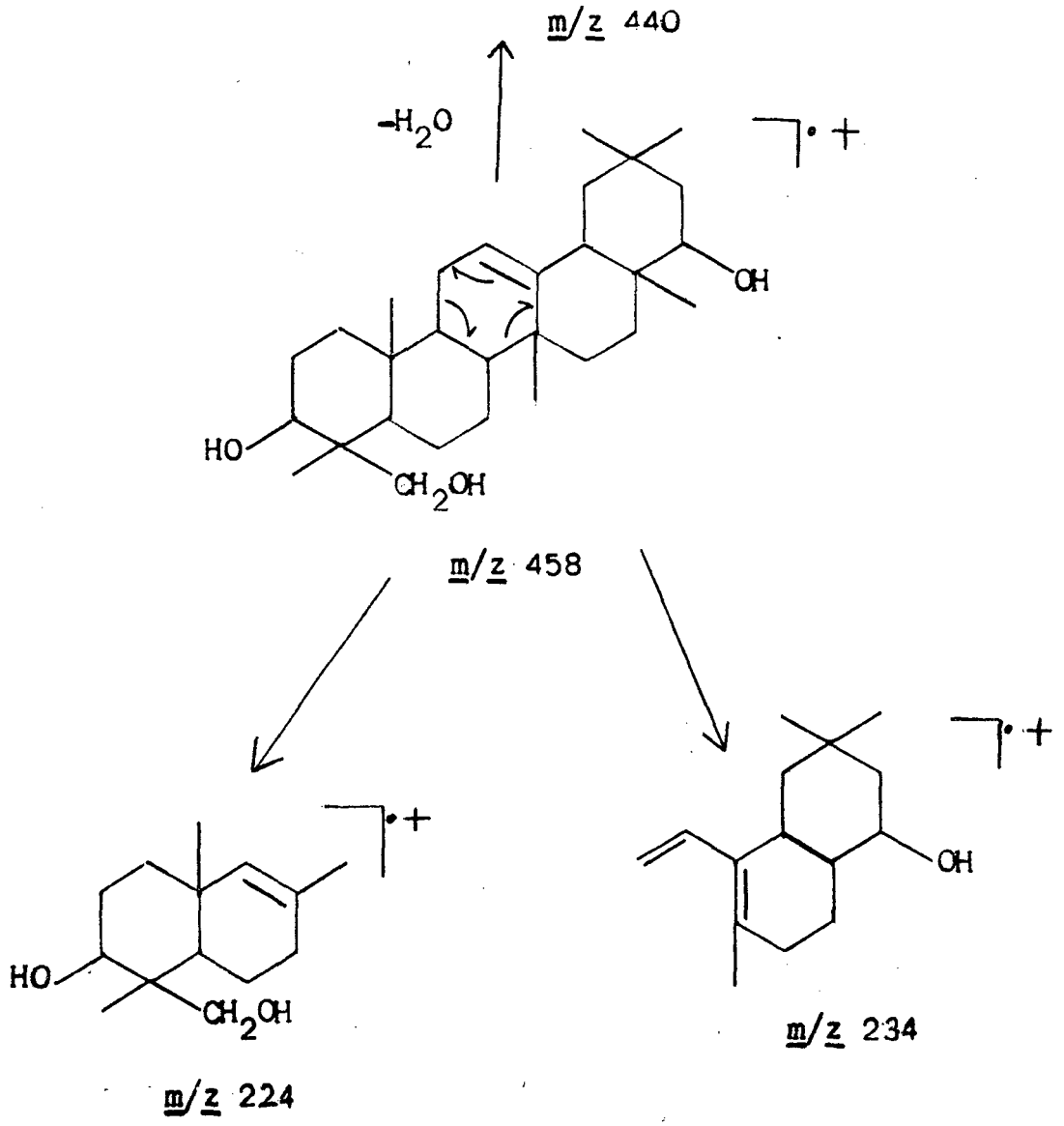


Figure 3

Substance G, m.p. 306°.

It was analysed for $C_{30}H_{48}O_3$ on the basis of its mass spectrum which displayed the molecular ion peak at m/z 456. It gave deep red colour in Libermann-Burchard test for the triterpenes[18]. The IR spectrum showed strong absorption bands at 3480 cm^{-1} for hydroxy group[11] and another strong absorption band at 1710 cm^{-1} for the presence of the carbonyl group while the absorption bands at 1640 and 835 cm^{-1} were indicative of a trisubstituted (>C=CH) double bond[12]. The UV spectrum did not show any absorption in the characteristic range for the conjugated system and ruled out the possibility of conjugation between the double bond (>C=CH) and carbonyl (>C=O) group. The mass spectrum fragmentation pattern of the molecule and appearance of prominent ion peaks at m/z 248 and 207, formed by the retro Diels-Alder fragmentation determined the substance G to be an analogue of oleanene series of triterpene[19]. The formal structure was built up by the information proyided by PMR spectrum which showed a bunch of signals at δ 0.76-1.20 for seven methyl groups integrating for twenty one protons, a one proton multiplet at δ 3.30 for the carbinolic proton at C-3, and another one proton multiplet at δ 5.30 (J=11.0 Hz) for the vinylic proton (>C=CH) at C-12. The polymethylene protons showed a broad hump between δ 1.30-2.00. The confirmatory

evidences were drawn from the mass spectral fragmentation pattern of the compound which showed prominent ion peaks at m/z 441 (M^{+}·-Me), 438 (M^{+}-H_2O) and retro Diels-Alder fragment ions, as shown in Figure 4, were indicative of the C-12 position of the (>C=CH) double bond. The mono-acetate of the compound (12) confirmed the presence of one hydroxyl group by showing the molecular ion peak at m/z 498 which corresponds to the incorporation of one acetyl group in the parent molecule. The methylation of Substance G with diazomethane indicated the presence of acid functionality in the molecule which was placed at C-28 in accordance with the mass spectral fragment ion of parent molecule which showed prominent ion peak at m/z 248 and 203. The PMR spectrum of the methyl oleanate (13) provided complimentary evidences for the presence of acid functionality which showed a three proton singlet at δ 3.60 for the methoxyl protons.

With these data in hand and a thorough comparison with the authentic sample in all respects (m.p., m.m.p., co-T.L.C., co-I.R. and $[\alpha]_D$), the identity of substance G was established as oleanolic acid (11).

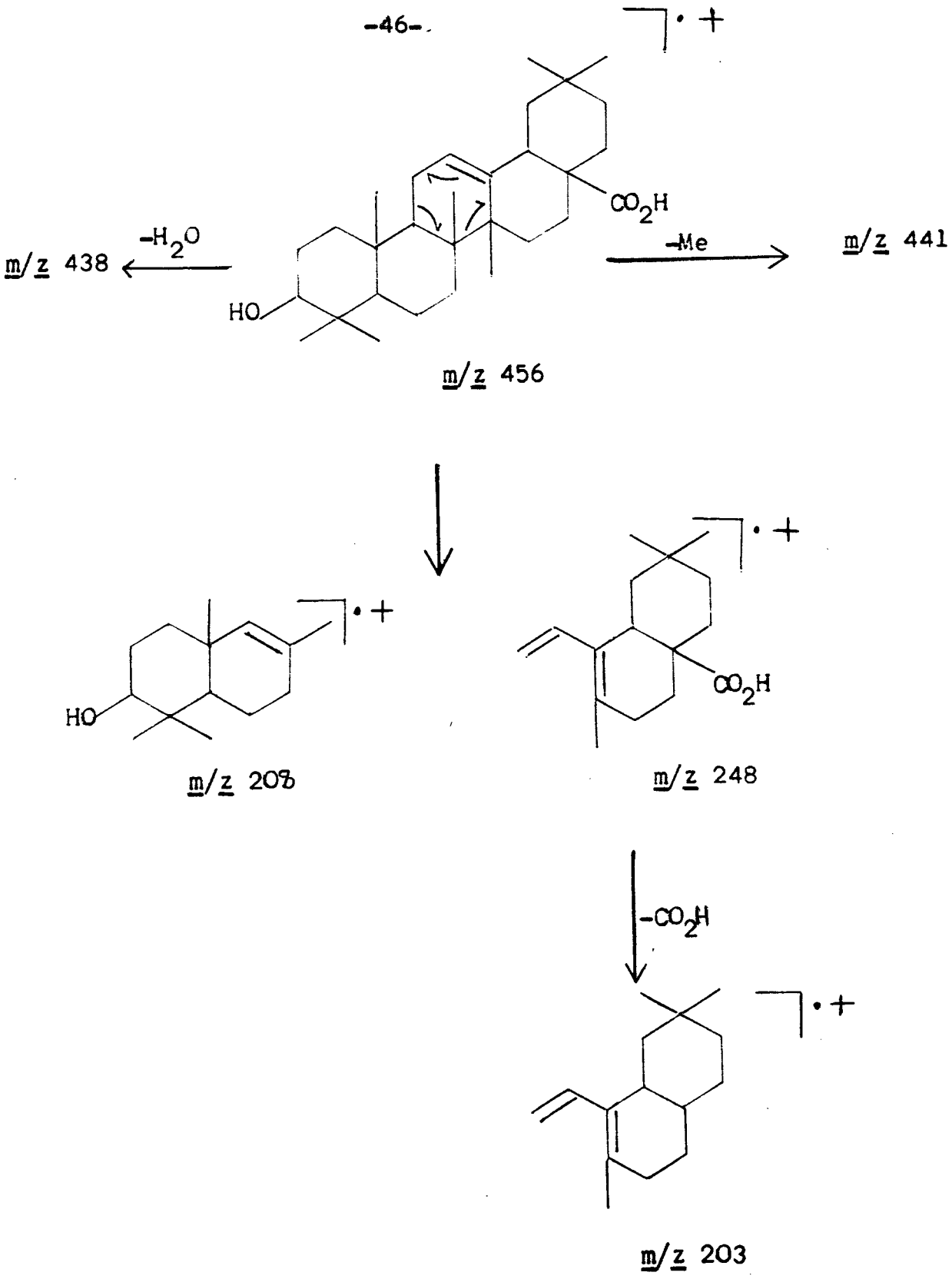

-H2O
m/z 438
HO
CO2H
-Me
m/z 441
m/z 456
HO
m/z 208
CO2H
m/z 248
-CO2H
m/z 203

Figure 4

Substance H, m.p.168°.

It was obtained as crystals from chloroform. The mass spectrum showed prominent molecular ion peak at m/z 424 for the assigned molecular formulae $C_{30}H_{48}O$. It responded to the Libermann-Burchard colour test[18] and gave a yellowish colour with tetranitromethane indicating the unsaturated triterpenoidal nature of the compound. In its IR spectrum it showed strong absorption bands at 1710, 1640 and 870 cm^{-1} for the presence of a carbonyl group and a substituted double bond. The sodium borohydride reduction of substance H (14) to an alcohol (15), which showed a strong absorption band at 3350 and 1040 cm^{-1} in its IR spectrum, further confirmed the presence of ketone moiety in the parent molecule. The exhibition of poorly resolved doublet at δ 4.6 (J=11.0 Hz) for two protons were accounted for the presence of vinylic proton in the parent molecule. The PMR spectrum also showed the presence of seven methyl groups at δ 0.80-1.20 integrating for twenty one protons and a multiplet at δ 2.40 was assigned to the methylene protons adjacent to the carbonyl group. The mass spectrum fragmentation pattern showed prominent ion peaks at m/z 424 (M^+), 381, 368, 355, 313, 257, 245, 218, 205 (base peak), 189 and 149 which was quite analogous to lupen-3-one. These foregoing data led to the structure (14) for substance H.

Substance I, m.p.216°.

It was obtained as white crystals from methanol. In its mass spectrum it showed the molecular ion peak at m/z 138 which well corresponds to the molecular formulae $C_7H_6O_3$. The observation of IR spectrum indicated absorptions for hydroxy group at 3400 cm^{-1} and carbonyl group at 1680 cm^{-1}. The UV spectrum showed the presence of benzene nucleus as the chromophore[26]. Further evidences were drawn from PMR spectrum which showed a two proton doublet at 7.60 (J=9.0 Hz) and another two proton doublet at 6.30 (J=9.0 Hz). The presence of acid moiety and hydroxyl group was followed by the mass spectrum fragmentation pattern which showed prominent ion peak at m/z 94 losing the CO_2 from the molecular ion. Further more the comparison of substance J with an authentic sample of p-hydroxybenzoic acid led to the confirmation of structure. The m.m.p., m.p., co-T.L.C. and co-I.R. ultimately established the identity of substance J as p-hydroxybenzoic acid (16).

Substance J, m.p.164°.

It was obtained as colourless slender blades from crystallization with ether in the butanol fraction of P.tuberosa. The UV spectrum displayed absorption peaks at 280 nm indicating the γ-pyrone nucleus of the compound[27]. The IR spectrum showed no significant

absorption peaks except for the presence of a conjugated carbonyl at 1665 cm^{-1}. The mass spectrum showed prominent molecular ion peak at m/z 126 which well corresponded to the molecular formulae $C_6H_6O_3$. The PMR spectrum revealed the nature of proton environment in the molecule which exhibited a three proton singlet at 2.40 for one methyl group which was highly deshielded by the presence of carbonyl moiety and one proton doublet at 7.60 (J=6.0 Hz) for the C-5 proton, located next to the carbonyl group and another one proton doublet at 6.40 (J=6.0 Hz) for the C-6 proton in the molecule. The diffused signal at 7.20 which appeared as a singlet integrating for one proton was disappeared on D_2O exchange experiment in the PMR spectrum could be ascribed to the hydrogen bonded hydroxy group. The tentative structure of γ-pyrone was followed as accomodating one methyl group and a hydroxy group located next to the carbonyl moiety. It also indicated the substitution pattern in the molecule and revealed the structure. These data were in complete accordance with the γ-pyrone structure 3-hydroxy-2-methyl-4H-pyran-4-one (Maltol) assigned to substance J (17).

The presence of maltol had been previously reported in the twigs of Pinus larix, Corydalis ochotensis, and several species of Abies[27]. It had also been found in roasted malt, coffee extract, wood tar and among the products of alkaline hydrolysis of streptomycin[28].

Substance K:

It was obtained as an amorphous solid in very low yield contaminated with substance L. The elemental composition of the substance K was determined to be $C_{15}H_{10}O_5$ on the basis of its mass spectrum which showed molecular ion peak at m/z 270. The UV spectrum followed the absorption maxima at 270 and 310 nm clearly indicating the isoflavone type of molecule[7]. The alkali induced shift in UV spectra at 275 and 315 nm further confirmed the presence of phenolic hydroxy groups in the compound. The detailed information was followed by the mass spectrum which indicated the presence of retro Diels-Alder fragment ion 'a' and 'b' in the mass spectrum at m/z 152 and 118, as depicted in Figure 5.

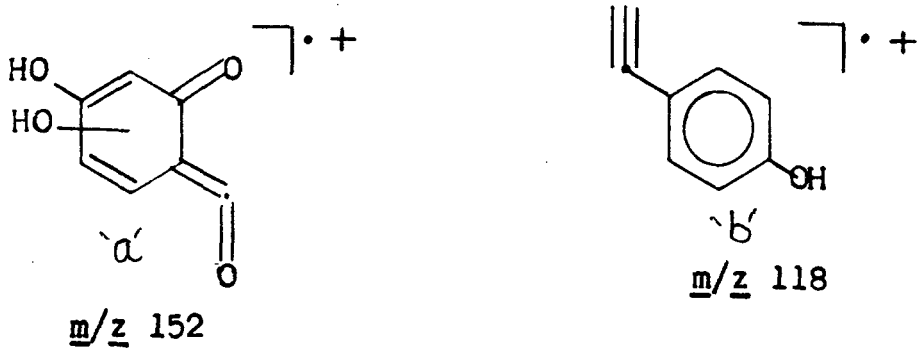


Figure 5: Retro Diels-Alder fragment ions for Substance K

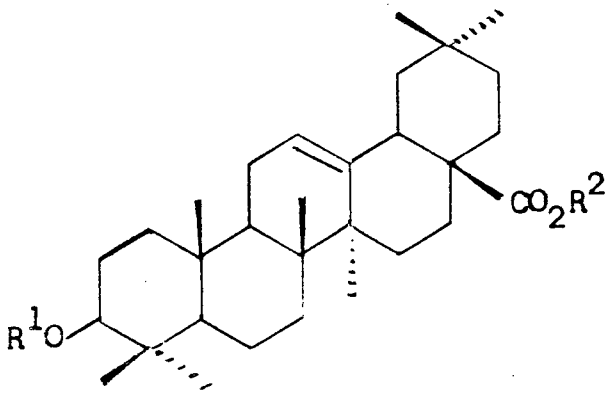
These daughter ions 'a' and 'b' helped in the placement of three hydroxy group, two in ring A and one in ring-B in the molecule. The one hydroxy group,

out of the two present in ring A, was placed at C-7 position in consideration with biogenetic origin[8]. However, the relative positions of these two hydroxy group could not be determined in absence of the PMR spectrum. The UV and mass spectra together provided the partial structure assigned for substance K. Thus, based on the available data substance K was designated as hydroxydaidzein (18).

The presence of iosmeric hydroxydaidzein had been previously reported from other natural sources. It is distributed in Glycine max[29], Phaseolus vulgaris[30] and heartwood of Machaerium villosum[31]. The former two sources contained 6-hydroxydaidzein, while the heartwood of Machaerium species yielded 3'-hydroxydaidzein.

Substance L: m.p.236°.

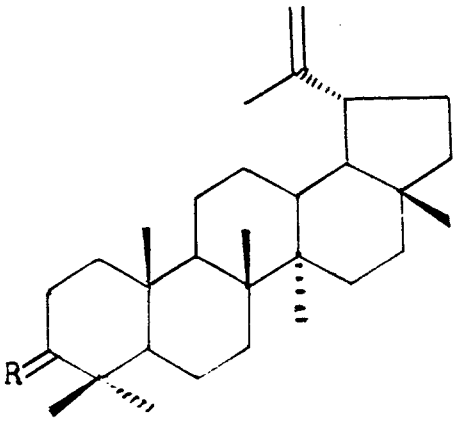
It was obtained on crystallisation from a mixture of ethylacetate-methanol. It exhibited only a weak ion fragment ion peak in El spectrum. The FD-MS of the compound confirmed the molecular ion peak at m/z 416 and elemental composition was determined to be as $C_{21}H_{20}O_9$. The UV spectrum displayed strong absorption maxima at 255 and 310 nm. It gave positive Molish's test which suggested the glycosidal nature of the compound[32]. The acid-hydrolysis of substance L with 6% dil hydrochloric



(11) R^1=H, R^2=H

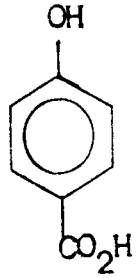
(12) R^1=Ac, R^2=H

(13) R^1=H, R^2=Me

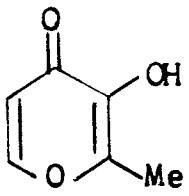


(14) R=O

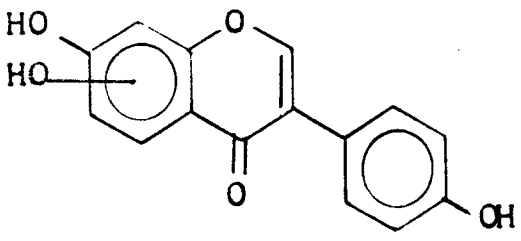
(15) R= 



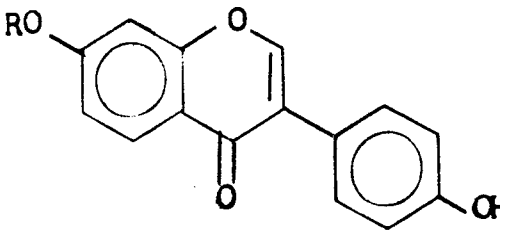
(16)



(17)



(18)



(19) R=Glu

(20) R=H

acid afforded daidzein (20) as aglycone moiety and glucose was identified as sugar by paper chromatography. It indicated that sugar was linked to the isoflavone moiety by a O-linkage and not through the C-linkage. Since the UV spectrum pattern of aglycone moiety resemble to that of daidzein and exhibited shift in UV spectrum on addition of alkali, in accordance with the presence of 4',7-dihydroxyisoflavone, the substance L was determined to be 4',7-dihydroxyisoflavone glucoside. The further structure elucidation was probed out with the help of PMR spectrum which showed six sugar C-H protons between δ 3.30-3.90, a broad singlet integrating for one proton at δ 5.10 for C_1-glucosyl proton. The pattern of aromatic substitution was deduced by the appearance of one proton doublet at δ 7.70 (J=8.5 Hz) for C_5-proton ortho to the carbonyl group and multiplets at δ 6.70 and δ 7.10 for the two proton in ring A and four protons in ring B collectively. However, the C-2 isoflavone singlet was observed at δ 8.05 integrating for one proton. All these foregoing data led to the assignment of structure (19): 7-O-β-D-glucopyranosyl-4',7-dihydroxyisoflavone (Daidzin) for substance L (19). The presence of daidzin had also been previously reported from the North American species of Thermopsis[33] and soybeans[34].

II.3 Experimental

All melting points were taken on an electrically heated block (Toshniwal Bros., Ltd.) and are uncorrected. The values are expressed in ^{o}C. The UV spectra were recorded on a Hitachi 320 instrument or on a Perkin Elmer Lambda-5 spectrophotometer. The IR spectra of the compounds were recorded in KBr pellets or neat on a Perkin-Elmer 577 instrument and the values (ν_{max}) are expressed in cm^{-1}. The PMR spectra were taken either at 90 MHz on Perkin-Elmer, R-32 instrument or at a 80 MHz on CFT-20 instrument. The spectra of the compounds were also recorded on 400 MHz Bruker WM-400 Fourier transform spectrometer using tetramethylsilane as an internal reference. The chemical shift values are expressed in δ units. The EI mass spectra were recorded on a Jeol D-300 spectrometer fitted with a direct inlet system at 70 eV.

Isolation of Constituents:

The freshly chopped tubers of P.tuberosa (100 kg) collected from Shahdol forest (M.P.) were extracted with 95% EtOH (2x50 l). The extract on evaporation of solvents below 50^{o} yielded a gummy solid material (1.2 kg) which was diluted with 2.0 litres of water and extracted with hexane (4x2 l) to afford the hexane soluble fraction

(7.0 g). The defatted material was again extracted with chloroform (4x2 l) and n-butanol (4x2 l) to give the chloroform soluble material (103.0 g) and n-butanol soluble material (72.5 g) respectively, after concentration under vacuo.

The fractionation scheme for P.tuberosa follows as in Chart 1.

Chromatography of Chloroform Soluble Fraction:

A part of the chloroform soluble fraction (50 g) was subjected to gross-fractionation on a column of silica gel in hexane. The column was eluted with hexane and then with the variable mixtures of hexane and ethyl acetate with increasing polarities. The fractions of 500 ml each were collected and mixed together on the basis of their T.L.C. pattern.

The chloroform soluble fraction on extensive chromatographic purification yielded different class of compounds viz. aliphatic acid, phytosterols, triterpenes and isoflavonoids including the previously known pterocarponoids which enlist anhydrotuberosin, 3-O-methylanhydrotuberosin, tuberostan, tuberosin and a novel fungal metabolite 1a-hydroxytuberosone.

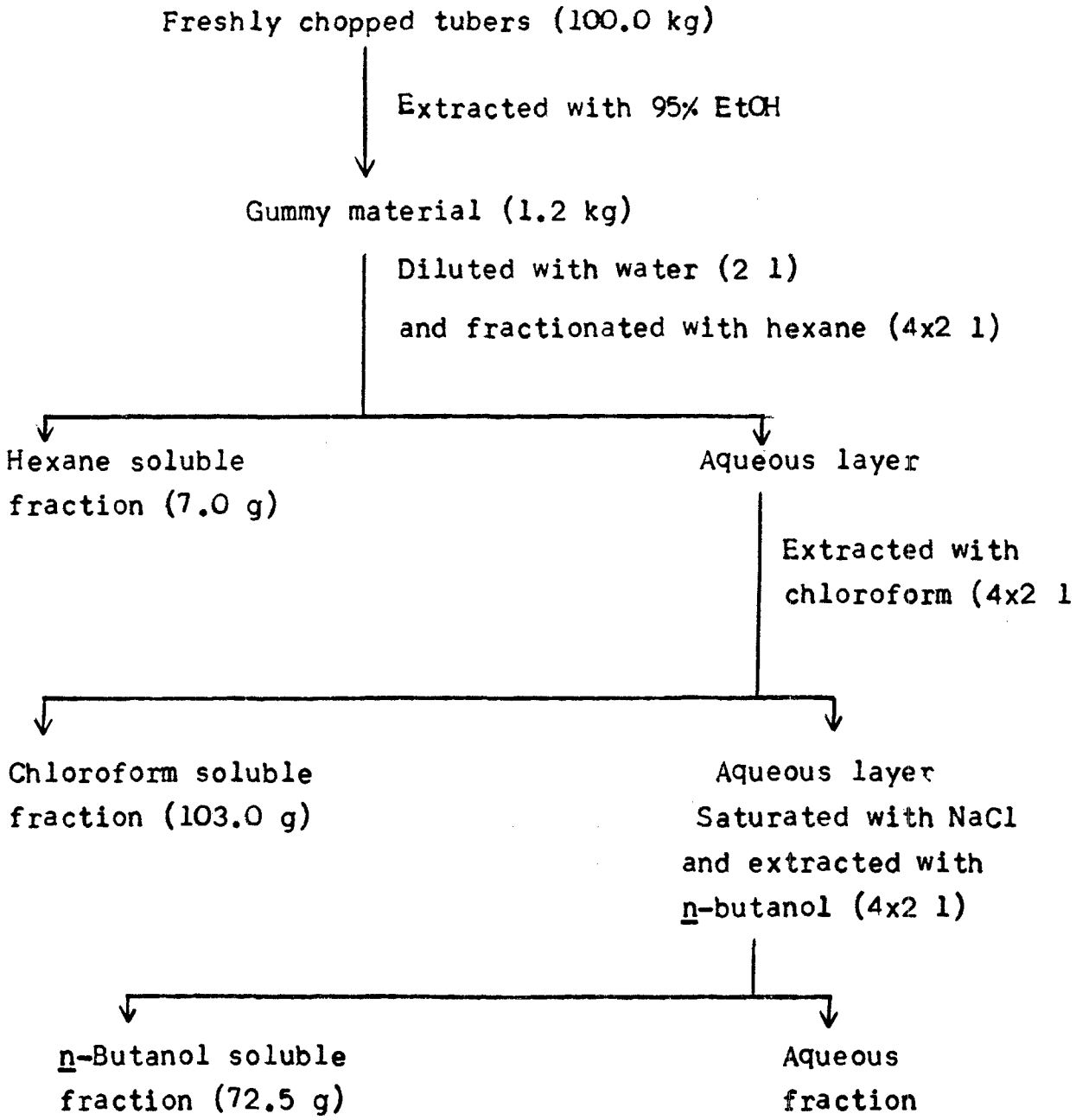


Chart 1: Fractionation Scheme of P.tuberosa.

The results of chloroform soluble fraction are summarised in Table 2.

Table 2: Column chromatography of chloroform soluble fraction

Fraction No.	Eluent	Remarks
1-7	Hexane	Oily mixture
8-11	Hexane : Ethyl acetate (90:10, v/v)	Substance A
12-16	Hexane : Ethyl acetate (85:15, v/v)	Substance A+B
17-21	Hexane : Ethyl acetate (80:20, v/v)	Substance C
22-26	Hexane : Ethyl acetate (75:25, v/v)	Substance C+D
27-31	Hexane : Ethyl acetate (70:30, v/v)	Substance E
32-37	Hexane : Ethyl acetate (65:35, v/v)	Substance E+F
38-43	Hexane : Ethyl acetate (60:40, v/v)	Substance G
44-50	Hexane : Ethyl acetate (55:45, v/v)	Substance H
51-56	Hexane : Ethyl acetate (50:50, v/v)	Streaking
57-61	Hexane : Ethyl acetate (50:70, v/v)	Streaking

Further purification of Substances A and B from the combined fractions:

The fraction (12-16) was flash chromatographed on a column of silica gel. It was eluted with hexane and a mixture of hexane and ethyl acetate (95:5, v/v). The fractions of 50 ml each were collected and examined on T.L.C. to yield the pure compounds A (10 mg) and B (30 mg).

Substance A (Isomedicarpene), m.p.133° (l).

UV(MeOH) : 280, 306 and 340 nm

(MeOH+NaOH) : 305, 350 and 355 nm.

IR(KBr) : 3400 (br, OH), 2900, 1610, 1080 and 1025 cm^{-1}

MS : 268 (M^+), 267 (M^+-1, base peak), 253, 224, 197 and 134.

PMR($CDCl_3$) : 3.70 (s, 3H, OMe), 5.40 (s, 2H, C_6-H), 6.35-7.20 (m, 5H, Ar-H) and 7.30 (d, 1H, J=9.0 Hz, C_1-H).

Acetylation of Substance A:

Substance A (5 mg) was acetylated with acetic anhydride (1 ml) and dry pyridine (1 ml) by keeping it overnight at room temperature and conversion was monitered by T.L.C. The solvent was removed under vacuo

to furnish an oily residue which was dissolved in chloroform, washed with water and dried over anhydrous Na_2SO_4 to afford an oily product (5.0 mg) (2).

MS : m/z 310 (M^+), 267, 197 and 134.

Substance B (β-Sitosterol), m.p.136°, $[\alpha]_D$-34.6°, (3).

IR : 3650 (OH), 2450, 1640, 1040, 835 and 800 cm^{-1}

MS : m/z 414 (M^+), 399, 396, 381, 329, 289, 273, 255, 231, 213, 133, 119, 95 and 81

PMR($CDCl_3$) : 0.62-0.96 (bunch of singlets,18H, 6 x Me), 1.20-1.84 (m, 22H, methylenes), 3.60 (m, 1H, CH-OH) and 5.44 (m, 1H, -C=CH-CH_2).

Substance C (Stigmastan-3-one), m.p.156.(4)

IR : 2950, 2870, 1710, 1460, 1250, 1900, and 870 cm^{-1}

MS : m/z 414 (M^+), 399, 273, 271, 229 and 203

PMR($CDCl_3$) : 0.70-1.10 (bunch of singlets,18H, 6 x Me), 1.20-1.95 (m, 20H, methylenes) and 2.20-2.50 (m, 4H, CH_2-CO-CH_2).

Substance D (β-Amyrin), m.p.197° $[\alpha]_D$ +88.0 ($CHCl_3$).(5)

IR : 3450 (OH), 1640, 1340, 1045, 840 and 780 cm^{-1}.

MS : m/z 426 (M^+), 398, 218 (base peak)

PMR($CDCl_3$) : 0.75-1.20 (bunch of singlets,24H, 8 x Me), 1.70-2.30 (m, 20H, methylenes), 3.30 (m, 1H, CH-OH) and 5.22 (m, 1H, >C=CH)

Acetylation of Substance D:

Substance D (10 mg) was acetylated with acetic anhydride (2 ml) and dry pyridine (2 ml) by keeping it overnight at room temperature and conversion was monitered by T.L.C. The solvent was removed under vacuo to furnish a residue which was dissolved in chloroform, washed with water and dried over anhydrous Na_2SO_4. The product was crystallized from hexane-chloroform to afford the acetate (12.0 mg) (6).

m.p. : 234°

MS : m/z 448 (M^+).

Purification of Substance E and F:

The fractions containing the mixture of Substance E and F were combined together and concentrated to furnish a gummy material which was subjected to further purification by flash chromatography in a solvent system

of hexane : ethyl acetate (4:1, v/v). The fractions were collected and examined on T.L.C. to yield the pure compounds. The compound E was detected under UV light which gave a green illuminance over UV sensitive silica gel (UV_{254}).

Substance E (Puerarone) oily material (7)

UV(MeOH) : 230 and 305 nm

(MeOH+NaOH) : 235 and 325 nm

IR(Neat) : 3400 (OH), 2900, 1660, 1450, 1240, 1050, 840 and 780 cm^{-1}

MS : m/z 336 (M^+), 321 [(M^+-Me), base peak], 185 and 136

PMR($CDCl_3$) : 1.37 (s, 6H, 2 x Me), 5.50 (d, 1H, J=10.0 Hz, $C_{1''}$-H], 6.30 (d, 1H, J=10.0 Hz, $C_{2''}$-H), 6.94 (d, 1H, J=2.5 Hz, C_8-H), 7.10 (dd, 1H, J=8.5, 2.5 Hz, C_7-H), 8.05 (d, 1H, J=8.5 Hz, C_5-H) and 8.25 (s, 1H, C_2-H).

Acetylation of Substance E:

Substance E (10.0 mg) was acetylated with acetic anhydride (2 ml) and dry pyridine (2 ml) by keeping it overnight at room temperature and conversion to acetate was followed by T.L.C. The solvent was removed under

vacuo to furnish a residue which was dissolved in chloroform, washed with water and dried over anhydrous Na_2SO_4 to afford an oily product (12.0 mg) (8).

MS : m/z 420 (M^+), 378 (M^+-Ac), 334, 185 and 136.

Substance F (Soyasapogenol-B), m.p.256°. $[\alpha]_D$ +92.0 ($CHCl_3$).(9)

IR : 3450 (OH), 1640, 1040, 980, 835 and 820 cm^{-1}

MS : m/z 458, 442, 234 (base peak), 224 and 176

PMR($CDCl_3$) : 0.90-1.24 (bunch of singlets, 21H, 7 x Me), 1.46-1.94 (m, 16H, methylenes), 2.82 (m, 1H, C_{22}-H), 3.26 (m, 1H, C_3-H), 5.28 (m, 1H, -C=CH_2, C_{12}-H).

Acetylation of Substance F:

Substance F (15 mg) was acetylated with acetic anhydride (5 ml) and dry pyridine (5 ml) by keeping it overnight at room temperature and the conversion was monitered by T.L.C. The solvent was removed under vacuo to furnish an oily material which was dissolved in chloroform, washed with water and dried over anhydrous

Na_2SO_4. The product was crystallised from chloroform to afford the acetate (20.0 mg) (10).

m.p. : 176°

MS : m/z 584 (M^+).

Substance G (Oleanolic acid), m.p.306°. $[\alpha]_D$+79.5° ($CHCl_3$) (11)

IR : 3480 (br, OH), 2950, 1700 (>C=O), 1270, 980, 820 and 790 cm^{-1}.

MS : m/z 456 (M^+), 441, 438, 248 (base peak), 203, 133, 95 and 81.

PMR($CDCl_3$) : 0.76-1.20 (bunch of singlets,21H, 7 x Me), 1.30-2.00 (m, 18H, methylenes), 3.30 (m, 1H, CH-OH), 5.30 (m, 1H, J=11.0 Hz, >C=CH).

Acetylation of Substance G:

Substance G (10 mg) was acetylated with acetic anhydride (2 ml) and dry pyridine (2 ml) by keeping it overnight at room temperature. The conversion to acetate derivative was followed by T.L.C. The solvent was removed under vacuo to furnish a residue which was dissolved in chloroform, washed with water and dried over anhydrous Na_2SO_4 to afford the product (10.0 mg) (12).

m.p. : 268°

MS : m/z 498 (M^+)

Methylation of Substance G:

A freshly prepared ethereal solution of diazomethane from N-methyl nitrosourea (50.0 mg) and ice cold solution of KOH (1.0 g) was added to the ethereal solution of substance G (20.0 mg). The content was left for 10.0 minutes in the ice bath. The solvent was removed to furnish a residue which on crystallisation with methanol-chloroform afforded colourless needles (15.0 mg) (13).

m.p. : 196°

MS : m/z 470 ($M^{+\cdot}$).

Substance H (Lupen-3-one), m.p.168°. $[\alpha]_D$ +63.0 ($CHCl_3$) (14)

IR : 2840, 1700 (>C=O), 1640, 1450, 1350, 1120, 985, 870 and 835 cm^{-1}

MS : m/z 424 (M^+), 409 (M^+-Me), 381, 368, 355, 313, 257, 245, 218, 205 (base peak) 189 and 149

PMR($CDCl_3$) : 0.80-1.20 (bunch of singlets, 21H, 7 x Me), 1.60 (s, 3H, CH_2=C(CH$_3$)-), 2.40 (m, 2H, >C=CH_2) and 4.60 (d, J=11.0 Hz, CH_2=C(CH$_3$)-).

Reduction of Substance H:

Substance H (20.0 mg) was dissolved in methanol (10.0 ml) and $NaBH_4$ (20.0 mg) was added to it in small portions. The stirring was continued for 3 hr. The conversion was followed by T.L.C. and after the completion of the reaction, the solvent was removed under vacuo to furnish a residue which was diluted with water, acidified with dil. hydrochloric acid and extracted with chloroform (2x10 ml). The combined chloroform layer was washed with water and dried over anhydrous Na_2SO_4 to afford the product (15 mg) (15).

m.p. : 215°

IR : 3350 (OH), 2930, 1620, 1450, 1210, 985, and 835 cm^{-1}

MS : m/z 426 (M^+).

Chromatography of n-butanol fraction:

The n-butanol fraction (25.0 g) was chromatographed over a column of silica gel in ethyl acetate. It was eluted with ethyl acetate followed by methanol with increasing polarity. The fractions of 500 ml each were collected and mixed together on the basis of T.L.C. The results on column chromatography of n-butanol fraction is summarised in Table 3.

The extensive purification of the n-butanol soluble mixture yielded the previously known compounds from the extract which enlist daidzein, dihydrodaidzein and puerarin as the major constituents.

Table 3: Column chromatography of n-butanol fraction

Fraction No.	Eluent	Remarks
1-3	Ethyl acetate	Substance I
4-7	Ethyl acetate : Methanol (90:10, v/v)	Substance J
8-12	Ethyl acetate : Methanol (80:20, v/v)	Substance K+L
13-18	Ethyl acetate : Methanol (70:30, v/v)	Substance L
19-25	Ethyl acetate : Methanol (50:50, v/v)	Streaking

The further purification of fraction containing substance K and L were chromatographed over a small column of silica gel yielding substance K (5 mg) and substance L (25 mg) in poor yield.

Substance I (p-Hydroxybenzoic acid), m.p.216°.(16)

IR : 3300, 1680, 1640, 1420, 1360, 970, 780 and 740 cm^{-1}

MS : m/z 138 (M^+), 121, 94 and 63

PMR($CDCl_3$) : 6.3 (d, 2H, J=9.0 Hz) and 7.6 (d, 2H, J=9.0 Hz)

Substance J (Maltol), m.p.164°. (17)

UV(MeOH) : 280 nm

IR : 1665 cm^{-1}

MS : m/z 126 (M^+), 111 and 94

PMR($CDCl_3$) : 2.40 (s, 3H, Me), 6.40 (d, 1H, J=6.0 Hz, C_6-H), 7.60 (d, 1H, J=6.0 Hz, C_5-H) and 7.20 (s, 1H, >C-OH, exchangeable).

Substance K (Hydroxydaidzein). (18)

UV(MeOH) : 270 and 310 nm

(MeOH+NaOH) : 275 and 325 nm

MS : m/z 270 (M^+), 152 and 118.

Substance L (Daidzin), m.p.236°. (19)

UV(MeOH) : 255 and 310 nm

(MeOH+NaOH) : 255 and 320 nm

IR : 3350 (br, OH), 1640, 1620, 1450, 1380, 1260, 1050, 880 and 835 cm^{-1}

EI-MS : m/z 254

FD-MS : m/z 416 (M^+), 397, 379, 361, 307, 254, 137 and 118

PMR($CDCl_3$+ DMSO-d_6) : 3.30-3.90 (m, 6H, glucosyl proton), 5.10 (brs, 1H, glucosyl H-1), 6.70 (m, 3H, C_3,-H, C_5,-H, and C_8-H), 7.10 (m, 3H, C_2,-H, C_6,-H and C_6-H), 7.70 (d, 1H, J=8.5 Hz, C_5-H) and 8.05 (s, 1H, C_2-H).

Acid Hydrolysis of Substance L:

Substance L(15.0 mg) was mixed with 6% aqueous hydrochloric acid (10.0 ml) using a minimum of methanol to effect complete solution. The solution was heated on steam bath for about one hour and then cooled and extracted thoroughly by shaking with ether. It was washed with water and evaporation of the aqueous layer under reduced pressure and below 50° temperature yielded glucose which was identified by comparison with an authentic sample by paper chromatography using n-butanol-acetic acid - water (6:1:3, v/v) as the solvent system. The ether layer was washed with water, dried over anhydrous Na_2SO_4 to yield daidzein (20) as the aglycone moiety.

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